

THE INTERACTION BETWEEN HUMAN MONOCYTES  
AND RED CELLS

BINDING CHARACTERISTICS\*

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Warm antibodies may induce severe hemolytic anemia, spherocytosis, and sequestration of red cells in the reticulo-endothelial system, primarily in the spleen (1). These antibodies are "incomplete;" they fix little or no complement and cause neither morphologic changes nor lysis of red cells in vitro.

That warm antibodies may affect red cells in vitro was first demonstrated by Jandl and Tomlinson in 1958, when it was observed that human red cells coated with anti-D antibody were bound circumferentially about a white cell in a "rosette" pattern (2). Bound red cells appeared spherocytic. Rosette formation has since been induced in a variety of experimental systems in which leukocytes from different species are incubated with heterologous or homologous red cells (2-10). Nevertheless, there remains some controversy regarding the nature of rosette-forming leukocytes, the specificity of the mononuclear receptor site, and the relationship of rosette formation to phagocytosis.

In a previous brief communication, evidence was presented which indicated that human mononuclear cells have specific surface receptors for IgG and suggested that these may provide a mechanism by which antibody-coated red cells may be apprehended and destroyed in vivo (5). We report here further studies on the specificity of the mononuclear receptor site for IgG globulins and the ultrastructural characteristics of red cell-mononuclear cell interaction.<sup>1</sup> In an accompanying paper (12), studies on the role of IgG subclasses and complement, as well as a possible peptide responsible for binding are described.

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<sup>1</sup> A portion of this work was presented to the American Federation of Clinical Research, Atlantic City, N. J., May, 1970, and reported in abstract form (11).

*Materials and Methods*

*Proteins.*—Anti-D sera were derived from patients with Rh incompatibility during pregnancy and from one individual who had been immunized previously to produce a high titer of anti-D. These anti-D antibodies were IgG globulin and caused no agglutination or complement fixation. Sera from normal type O individuals were used for anti-A and anti-B isoagglutinins. The Donath-Landsteiner antibody was obtained from the serum of a patient with paroxysmal cold hemoglobinuria.<sup>2</sup> Antibody eluates were prepared from red cells of patients with immunohemolytic anemia according to the method of Weiner (13). Elution from DEAE-cellulose column chromatography at 0.005 M phosphate buffer, pH 8.0, was used to obtain IgG from normal human or chimpanzee serum. IgM was isolated from the serum of a patient with Waldenström's macroglobulinemia (14).

*Cells.*—Human white cells were obtained by dextran sedimentation (½ volume of 3% dextran in saline) of heparinized peripheral blood (100 USP units of heparin/ml of whole blood). The white cell-rich supernatant was centrifuged at 100 g for 12 min at 4°C. The cell button was resuspended with Hanks' balanced salt solution (containing  $5 \times 10^{-4}$  M MgCl<sub>2</sub> and  $1.3 \times 10^{-3}$  M CaCl<sub>2</sub>)<sup>3</sup> to a white cell count of 10,000–20,000/mm<sup>3</sup>. All glassware was siliconized. White cells from the chimpanzee and pigtail monkey were similarly obtained by dextran sedimentation. White cells of the rhesus monkey were harvested after sedimentation with 5% bovine fibrinogen.<sup>4</sup> Human blood was defibrinated or anticoagulated with acid-citrate-dextrose (ACD).<sup>5</sup> For radioactive labeling, Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (50–150 μCi/ml whole blood) was employed according to standard techniques(1).

*Attachment of Protein to Red Cells.*—Equal volumes of washed (80–100 volumes of Hanks') normal red cells at a final concentration of 50 volumes per cent, and serum antibody or red cell eluate containing warm antibody were incubated for 1 hr at 37°C, after which the cells were rewashed in Hanks' solution. Protein was also coupled to washed red cells using chromic chloride (15); to a mixture containing one part red cells (defibrinated blood washed six times with saline and brought to a cell concentration of 50 volumes per cent) and five parts of a solution of protein in saline (1 g/100 ml), 2 parts of chromic chloride (Cr<sup>+++</sup>) solution in saline (5 μmoles Cr<sup>+++</sup>/ml red cells for the coupling of IgG, and 30 μmoles Cr<sup>+++</sup>/ml red cells for albumin) were added. After 4 min at room temperature, the cells were washed four times with saline and tested with monospecific Coombs serum to confirm the coupling of protein (16). Cold hemolysins (heated at 56°C for 30 min in order to inactivate complement) and cold agglutinins were placed on red cells in standard fashion (16).

*Incubation Technique.*—To a vinyl cup<sup>6</sup> (⅝ inch diameter), 0.4 ml of the white cell suspension (10,000–20,000 white blood cells (WBC)/mm<sup>3</sup>) and 0.1 ml of Hanks' were added. After 45 min at 24°C, unattached cells were washed free with four washes of Hanks', leaving an adherent layer of granulocytes and mononuclear cells. Test red cells, at a cell concentration of 5 volumes per cent in Hanks', were placed over the monolayer and incubated for 2 hr at room temperature, at which time nonadherent red cells were removed by four washes with Hanks'. Rosette-inhibition experiments were performed by adding the test protein to the monolayer before the addition of red cells coated with anti-D. For radioactive counting, red cells were lysed with water directly from the Petri dish. Rosettes as well as noninvolved white cells could be detached from the plastic surface by incubation for 30 min with ethylenediaminetetraacetic acid (EDTA), 0.2 g/100 ml, in isotonic phosphate buffer, pH 7.4. For light microscopy,

<sup>2</sup> Kindly provided by Dr. W. Rosse.

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Petri dish preparations or detached cells were allowed to dry in phosphate-buffered saline containing albumin, 2 g/100 ml, and were stained with Wright's solution.

*Electron Microscopy.*—Two procedures were employed: (a) Suspensions of rosettes that had been detached by EDTA were centrifuged for 5 min at 100 *g* and resuspended in glutaraldehyde, 2.5 g/100 ml, in 0.1 M cacodylate buffer at pH 7.4 containing 0.1% calcium chloride. The cells were fixed for 1–2 hr at room temperature, with periodic shaking. They were then washed in cacodylate buffer overnight, postfixed in osmium-collidine, 1.3 g/100 ml, at pH 7.4 for 90 min, dehydrated in increasing concentrations of alcohol, infiltrated with 1:1 propylene oxide epon mixture, and imbedded in the bottom of conical polypropylene centrifuge tubes<sup>7</sup> or BEEM capsules.<sup>8</sup> Thin sections were cut on an LKB ultratome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate and viewed with a Philips EM 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.). (b) In some experiments leukocytes were incubated with red blood cells in vinyl cups. After incubation the rosettes were fixed *in situ* in glutaraldehyde, 2.5 g/100 ml, in cacodylate buffer. Subsequently the entire procedure of postfixation in osmium, dehydration, and imbedding was performed in the vinyl cups according to the method of Anderson and Doane (17). After polymerization of the epon, the vinyl was stripped from the polymerized block; areas containing rosettes were selected by phase microscopy, marked with a diamond pencil, and thin sections of the selected parts cut for electron microscopy.

Some preparations were tested for peroxidase activity because the peroxidase reaction allowed differentiation between neutrophils and mononuclears by light and electron microscopy. Glutaraldehyde-fixed, washed suspensions of cells were incubated in the diaminobenzidine H<sub>2</sub>O<sub>2</sub> medium of Graham and Karnovsky (18) for 15–30 min, washed, and for electron microscopy, postfixed in osmium as described above. By this method the large "azurophilic" granules of neutrophils were strongly stained (19), whereas the monocytes were either unstained or exhibited fine stippling by light microscopy and staining of smaller granules by electron microscopy (see below).

## RESULTS

I. *Antibody specificity for rosette formation.*—When normal red cells were coated with IgG antibodies and incubated over a monolayer of leukocytes from human peripheral blood, the antibody-coated red cells adhered to a central white cell in a rosette pattern (Fig. 1). Of the various IgG red cell antibodies, anti-D was most efficient in causing rosette formation, as measured by the amount of <sup>51</sup>Cr-labeled red cells bound to the monolayer. Antibodies associated with  $\alpha$ -methyl dopa, L-dopa, and penicillin administration as well as antibodies from idiopathic acquired (warm type) hemolytic anemia and paroxysmal cold hemoglobinuria were less efficient in forming rosettes but nevertheless the morphology of the rosettes was the same. When incubated over a monolayer in the absence of complement, red cells coated with IgM antibodies (anti-A, anti-B, or anti-I cold agglutinins) were not bound by white cells. In the presence of complement, IgM-coated red cells formed mixed agglutinates with white cells and the process was accompanied by phagocytosis of some of the red cells. Thus, IgG antibodies, which are frequently encountered

<sup>7</sup> Nalgene, Rochester, N. Y.

<sup>8</sup> Better Equipment for Electron Microscopy, Bronx, N. Y.

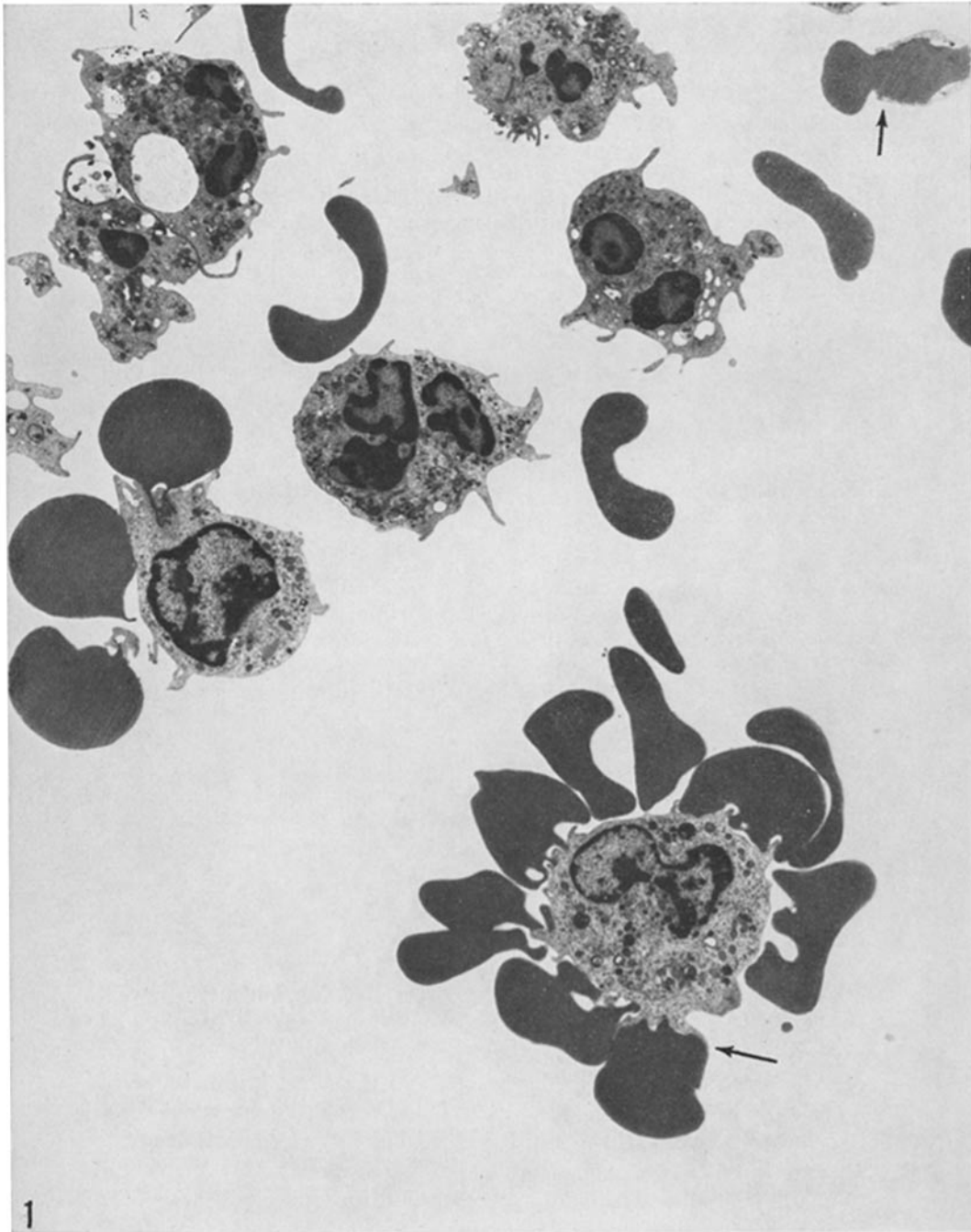


FIG. 1. Composite survey micrograph showing rosettes and unattached cells. The rosette-forming leukocytes are mononuclear cells while leukocytes without attached red cells are all neutrophils. Note the pseudopods extending from the monocytes around an attached red cell in a cup-shaped fashion (arrow). The attached red cells are deformed, while unattached erythrocytes have normal shapes.  $\times$  approximately (app.) 3500.

in immunohemolytic anemias in man, induced red cell binding; IgM antibodies produced complement-dependent mixed agglutination and phagocytosis.

Normal red cells coated with albumin by means of chromic chloride did not bind to mononuclear cells, whereas red cells similarly coated with normal (nonimmune) IgG readily formed rosettes. Transferrin, naturally present on the surface of reticulocytes, did not induce rosette formation.

II. *Rosette formation with primate leukocytes.*—Leukocytes from higher and lower primates were incubated with human red cells coated either with anti-D (Fig. 2) or with purified IgG globulin coupled by chromic chloride. Mononuclear cells from lower primates (rhesus and pigtail monkeys) formed very few rosettes; leukocytes from a higher primate (chimpanzee) formed many typical rosettes. However, 50% of the rosettes formed by the white cells of

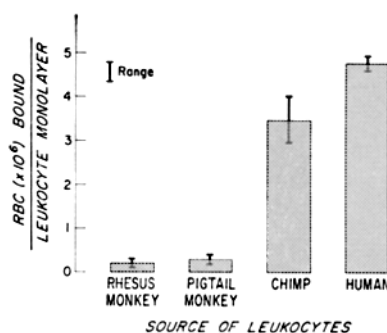


FIG. 2. The binding of anti-D-coated human red cells to the white cells of human and nonhuman primates. Red cells were trace-radiolabeled with  $\text{Na}_2^{51}\text{CrO}_4$ . Attachment of red cells to white cells was expressed as the number of red cells bound per monolayer.

the chimpanzee were polymorphonuclear leukocytes and, in striking contrast to the human system, phagocytosis was common. Purified IgG from the chimpanzee when coupled to red cells and compared with coupled human IgG bound less well to human white cells. However, when purified IgG from the chimpanzee was coupled to chimpanzee or human red cells by  $\text{Cr}^{+++}$  and tested with white cells of the chimpanzee, typical rosettes with mononuclear cells were formed.

III. *Rosette formation with abnormal red cells not coated with IgG.*—Leukocyte preparations were studied for their ability to bind red cells which possessed a variety of membrane or intracorpuseular defects. Red cells from patients with hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria, and sickle cell disease did not form rosettes. No rosette formation occurred with red cells altered by heating (20), storage (21), or sulfhydryl blockade (22).

IV. *Effect of chemical treatment on rosette formation.*—Pretreatment of white cells with cysteine-activated papain or with trypsin (0.05–5.0 g/100 ml) did not diminish the binding ability of monocytes. However, a variety of sulfhydryl-

binding reagents ( $2.4 \times 10^{-3}$  M *n*-ethyl maleimide,  $3.5 \times 10^{-3}$  M *p*-hydroxymercurobenzoate, and  $5 \times 10^{-3}$  M iodoacetate) did prevent the subsequent binding of anti-D-coated cells. KCN, NaF, or dinitrophenol did not cause binding or inhibition in concentrations as high as  $10^{-3}$  M. Hydrocortisone phos-

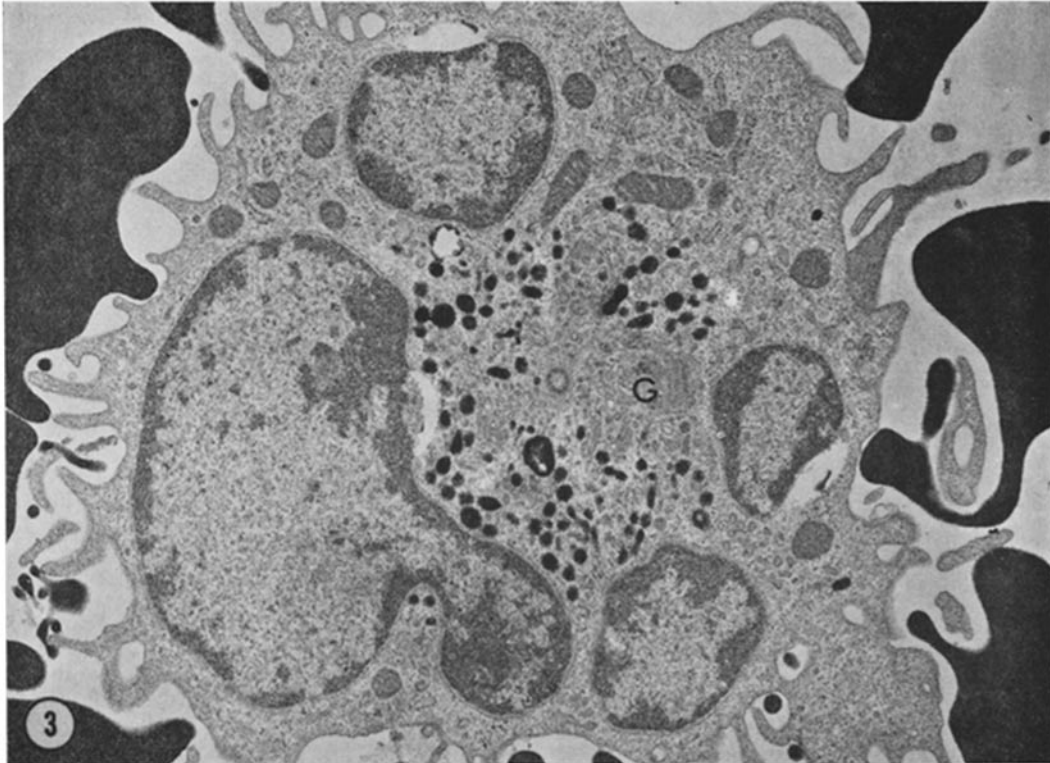


FIG. 3. Enlarged rosette-forming mononuclear cell from a preparation reacted for peroxidase activity according to Graham and Karnovsky (18). Note the large numbers of peroxidase-positive granules in the region of the Golgi complex (G); positive reaction in attached red cells is due to their hemoglobin content.  $\times$  app. 30,000.

phate (from 1 to 100 mg/100 ml) and heparin did not affect the binding process. Chloroquine (from 0.5 to 25 mg/100 ml) added to the leukocyte monolayer before or after the addition of anti-D-coated red cells did not affect rosette formation. In addition, a leukocyte monolayer prepared from a patient receiving oral chloroquine phosphate (500 mg/day) readily formed rosettes with anti-D-coated red cells.

Once rosettes were formed, neither sulfhydryl-binding agents nor 2-mercapto-

ethanol caused the release of the red cells. However, bound red cells could be detached by incubation with cysteine-activated papain at 37°C for 1 hr.

V. *Ultrastructural Studies.*—(a) *Identification of rosette-forming leukocytes:* The majority of rosette-forming cells in all preparations were mononuclear cells (Fig. 1). The most common were monocytes having an indented nucleus, abundant cytoplasm containing vesicles, heterogeneous dense granules, and

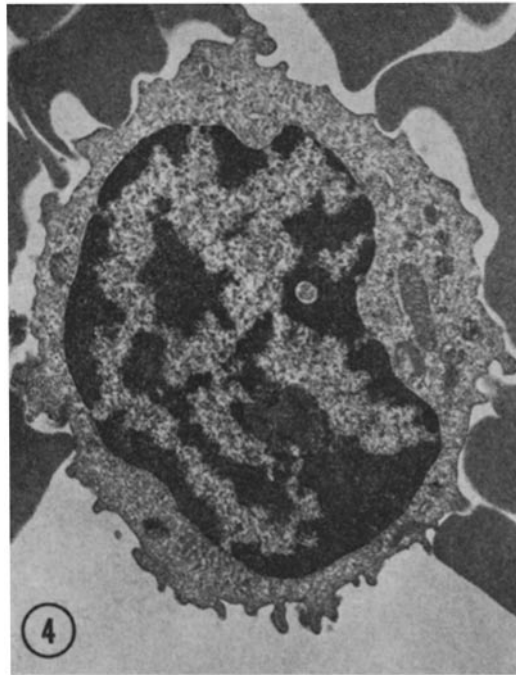


FIG. 4. This rosette-forming cell has a nucleolus, scanty endoplasmic reticulum, rare granules, and free ribosomes, and is interpreted as a cell of the lymphocyte series.  $\times$  app. 10,000.

aggregates of rough endoplasmic reticulum. A second common type of rosette-forming cell (Fig. 3) exhibited a horseshoe-shaped nucleus, prominent golgi apparatus, numerous small granules in the region of the golgi complex, fibrils, and large quantities of rough endoplasmic reticulum. The granules in the region of the golgi were positive for peroxidase activity by the Graham and Karnovsky technique (Fig. 3). Such cells were interpreted as "activated" monocytes, since they exhibited features similar to transformed peritoneal macrophages (23).

Some rosette-forming cells were characteristic of cells in the lymphocyte series (24), with a round or slightly oval nucleus, a small but sometimes prominent nucleolus, scant cytoplasm with free ribosomes, absent or rare granules,

and infrequent cisternae of rough endoplasmic reticulum (Fig. 4). Many cells had features which were intermediate between lymphocytes and monocytes. Neutrophils forming definite rosettes were rare. Neutrophils occasionally con-

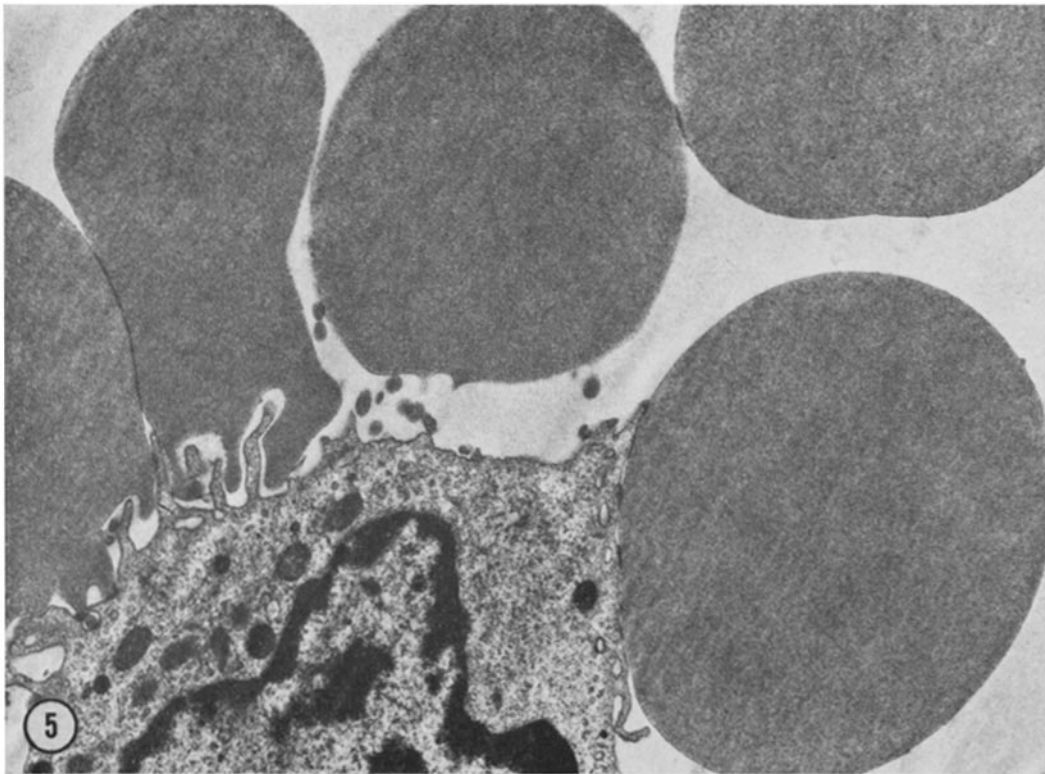


FIG. 5. Portion of a monocyte from a preparation of leukocytes incubated for 2 hr with anti-D-coated cells. On the left are the typical interdigitations between finger-like projections of monocyte and erythrocyte membranes, and on the right is an erythrocyte exhibiting zones of attachment that appear to be regularly spaced.  $\times$  app. 14,000. A portion of this micrograph has been published previously (5).

tained phagocytized red cells within their cytoplasm with two or three adherent erythrocytes.

The types of rosette-forming cells in one preparation were 70% monocytes 10% lymphocytes, 15% intermediate cells, and 5% granulocytes.

(b) *Attachment sites:* In any given plane of section as many as 15 red cells were attached to the cell membrane of the leukocyte. The sites of adherence exhibited a variety of forms. The most common was a complex interdigitation



between short villous processes from the monocyte with indentations in the erythrocyte membrane (Fig. 5). Often long delicate fingerlike projections from the monocytes, approximately  $1 \mu$  in thickness, appeared to grasp the red cells over extensive areas, sometimes entirely enveloping the intact cell (Fig. 6). Some attachment sites consisted of plaques of intimate contact between the

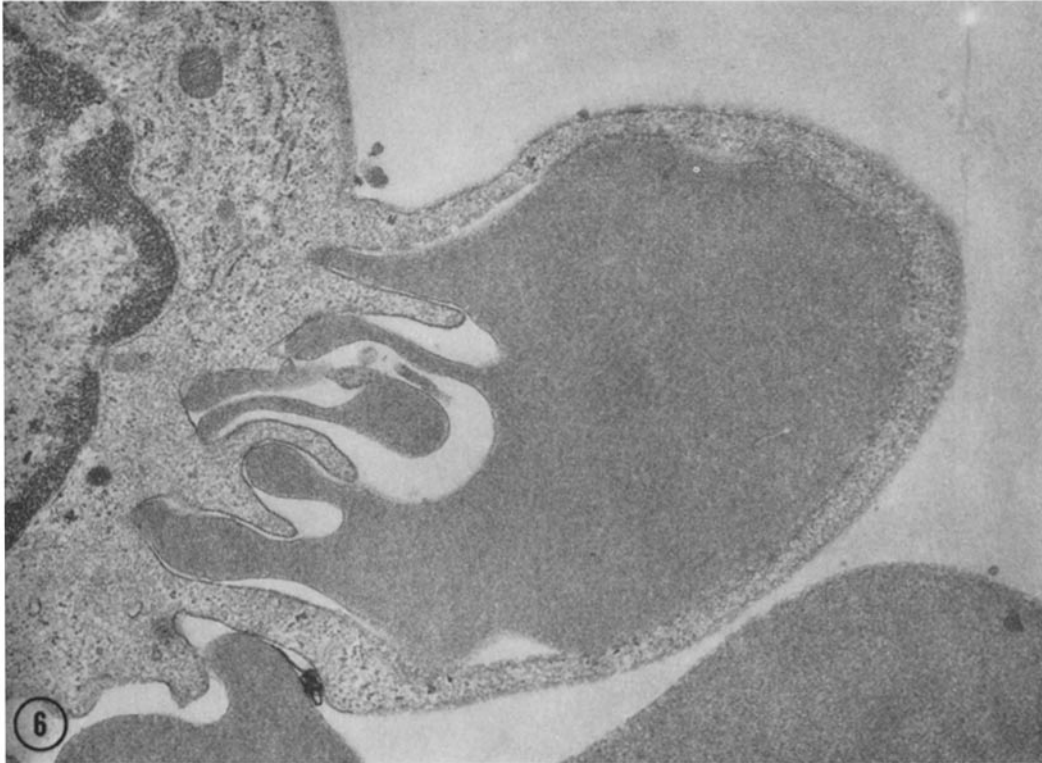


FIG. 6. Portion of a rosette from the same preparation as Fig. 3. The coated red cell has been completely enveloped by an extension of monocyte cytoplasm.  $\times$  app. 25,000.

erythrocyte and monocyte membrane; these plaques were sometimes regularly spaced (Fig. 5). A gap was usually present between the monocyte and the erythrocyte membrane. The width of this gap varied considerably and was probably due to variations in contraction during processing of the tissues. However, when cells were fixed in solutions of glutaraldehyde in phosphate buffer of varying osmolarities (from 200–600) there were no consistent differences in the widths of the gaps between cells. In rare instances there was approximation or apparent fusion of the external leaflets of erythrocyte and

monocyte membranes (Fig. 7). Some phagocytosis of red cells by monocytes and granulocytes occurred in most preparations, but the number of attached red cells greatly outnumbered phagocytized cells even after 4 hr of incubation.

(c) *The fine structure of attached erythrocytes:* In most instances attached red cells underwent considerable distortion and deformity in the region of attachments. Attached red cells appeared deformed, irregular, dense, and spherical (Fig. 1). Unattached red cells remained biconcave. Small rounded

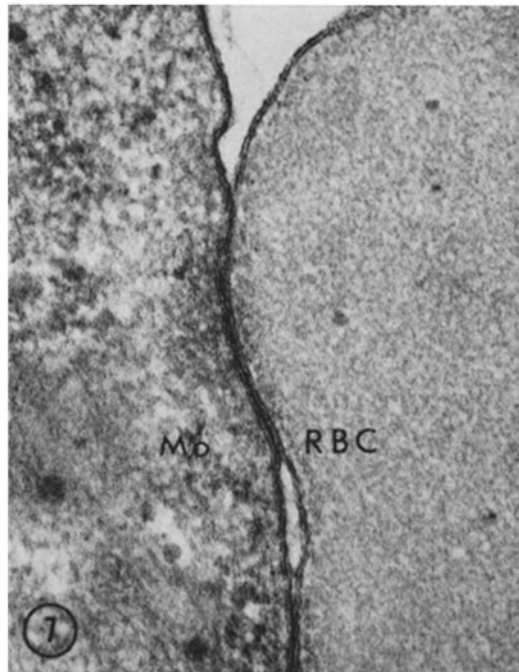


FIG. 7. Higher magnification of the zone of attachment between monocyte (MO) and red blood cell (RBC). At this site of attachment there is extreme approximation and apparent fusion of the external leaflets of contiguous cells.  $\times$  app. 110,000.

structures resembling red cell fragments (Figs. 3 and 5) were often seen apparently free in the space between the attached red cells and mononuclear cell, but because of the considerable interdigitation such fragments may have been continuous with the main red cell mass. Red cells when detached with papain were sometimes seen as deformed and irregular in shape and size by electron microscopy. Although deformities can be seen by electron microscopy as a result of the preparation, phase contrast microscopy on wet preparations confirmed the occasional morphologic abnormalities of red cells.

(d) *Erythrocytes coated with IgG globulin-Cr<sup>+++</sup> complexes:* Chromic chlo-

ride, when complexed to IgG globulin and coupled to red cells, was clearly visible by electron microscopy as a dense deposit adherent to the outer surface of the red cell membrane (Fig. 8). The ultrastructural characteristics of these rosettes (erythrocytes, leukocytes, and attachment sites) were similar to those described for anti-D-coated cells. The electron-opaque deposits on the red cell membrane persisted even after red cells or fragments were apparently ingested

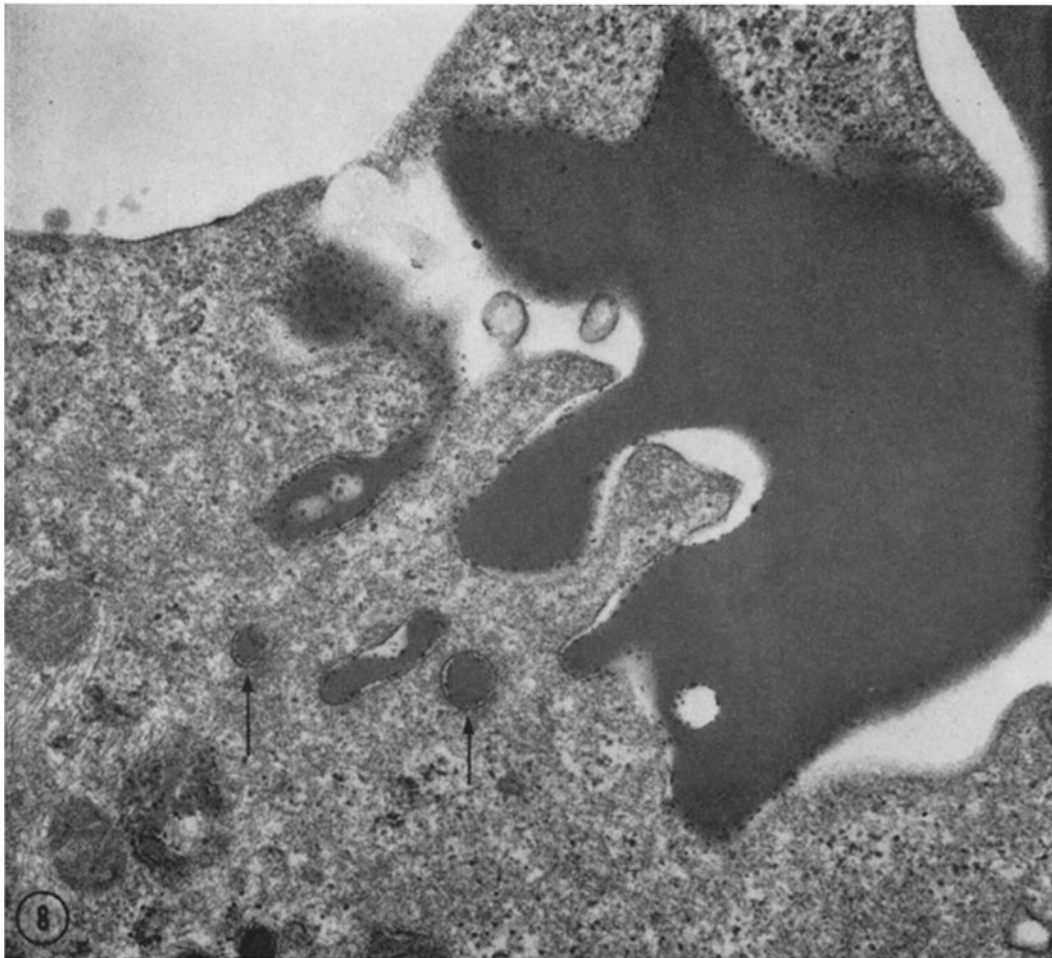


FIG. 8. Portion of a rosette from a preparation of human leukocytes incubated with red blood cells coated with IgG by means of chromic chloride. The chromic chloride-IgG complexes can be seen as electron-opaque deposits on the surface of the erythrocyte. Note that the hemoglobin-filled fragments within the cytoplasm of the leukocyte are surrounded by electron-opaque deposits (arrows).  $\times$  app. 30,000.

by the monocyte and facilitated the identification of hemoglobin-filled deposits within the cytoplasm of the leukocyte.

#### DISCUSSION

The findings reported in this study confirmed that certain human mononuclear cells possessed specific surface-receptor sites for IgG globulins. Different types of IgG red cell antibodies were capable of inducing rosette formation. These included noncomplement-fixing warm-active antibodies such as anti-D, anti-penicillin, and red cell antibodies from patients receiving  $\alpha$ -methyl-dopa, as well as cold-active complement-fixing IgG such as the Donath-Landsteiner antibody. The fact that fewer rosettes formed with eluates from idiopathic acquired hemolytic anemia may be related to less antibody activity in the eluates or to the relative distribution of IgG subclasses (11). In the second paper of this series (12) it will be shown that red cells coated with IgG<sub>1</sub> and IgG<sub>3</sub> formed rosettes whereas those coated with IgG<sub>2</sub> and IgG<sub>4</sub> did not.

The mechanism of rosette formation in the present experiments appeared to differ from that in heterologous systems in which "cytophilic antibody" attaches red cells to peritoneal macrophages at low temperature and in the absence of complement (4). In such animal systems, phagocytosis was observed at 37°C and lysis was noted in the presence of complement (4). Both guinea pig macrophages and human monocytes required free sulfhydryl groups for rosette formation. In contrast to the peritoneal macrophages of lower animals (8, 25), human monocytes were unable to bind red cells modified by storage, heating, or chemical treatment. Monocytes do react with glutaraldehyde-treated red cells (25); however, binding of such red cells differs from that of antibody-coated cells in two respects: (a) the attachment is not inhibited by IgG globulin, and (b) other types of white cells, such as neutrophils, form rosettes. Further, purified preparations of human monocytes derived from peripheral blood by centrifugation in an albumin gradient and grown in tissue culture for 24-72 hr do not demonstrate any activity with stored, heated, or sulfhydryl-treated red cells, but do demonstrate typical binding of IgG globulin-coated red cells.<sup>9</sup> Whereas phagocytosis is dependent upon glycolysis and is inhibited by fluoride and iodoacetate (23, 26), neither fluoride nor cyanide impaired rosette formation. Although chloroquine induces abnormalities of the granules in white cells (27) and partially impairs phagocytosis of sensitized red cells (28), it also had no effect on rosette formation.

The phylogeny of rosette formation was studied with white cells from other primate species. Maximal rosette formation with anti-D-coated cells occurred with human white cells; a lesser number of rosettes were noted with white cells of another higher primate (chimpanzee), and few rosettes occurred with

<sup>9</sup> Roth, G., A. F. LoBuglio, and J. H. Jandl. 1967. Unpublished observation.

white cells of lower primates (rhesus and pigtail monkeys). Of possible significance in this regard is the fact that human and chimpanzee possess the Rh locus, whereas pigtail and rhesus monkey do not. However, the binding of red cells coated with human anti-D occurred to both the mononuclear cells and granulocytes of the chimpanzee, and in addition, phagocytosis was common. On the other hand, red cells coated with purified IgG from the chimpanzee formed typical rosettes with a chimpanzee leukocyte monolayer. Therefore, it is more likely that rosette formation occurred best in homologous systems.

The predominant peripheral white cells involved in the formation of rosettes were blood monocytes. Although lymphocytes have not been reported to possess receptor sites for the Fc fragment of IgG globulin, some lymphocytes did form rosettes with anti-D-coated red cells, with red cells coated with antibody from patients with immunohemolytic anemias, and with IgG globulin coupled to red cells. However, lymphocytes comprised only 10% of the total rosette-forming population of white cells. Although these cells were lymphocytes by electron microscopic criteria, they were atypical of lymphocytes in that they adhered to the plastic dishes. Thus, mononuclear cells, which included monocytes, "sticky" lymphocytes, and cells which were intermediate between lymphocytes and monocytes accounted for approximately 95% of the rosette-forming cells as determined by electron microscopy (and light microscopy). Polymorphonuclear white cells rarely formed rosettes, and in those that did only a few adherent red cells were noted. In addition, phagocytosis of antibody-coated red cells by polymorphonuclear leukocytes was often seen even in the absence of complement.

A variety of red cell morphologic abnormalities were observed as a result of rosette formation. Frequently, the bound red cells appeared deformed. Some red cells, when detached by papain, remained deformed. "Fragments" of red cells were observed in the cytoplasm of the leukocyte and in the vicinity of the attachment sites. It was difficult to exclude that the fragmentation which was observed was an artefact of the extensive interdigitation between erythrocyte and leukocyte. Evidence that an actual loss of membrane occurred is suggested by an increased osmotic fragility (5), a measurement which depends on the surface to volume ratio; however, lipid determinations on attached red cells have not yet been performed. Brabec et al. (29) have reported a decrease in red cell lipids in patients with warm antibody-induced hemolytic anemia.

Red cell attachment to monocytes, as reflected by rosette formation, may have significance in the pathophysiology of immunohemolytic anemias. In the absence of the mononuclear cell, IgG globulin does not cause any morphological alteration to red cells. In vivo the mononuclear cell, as it resides in the form of a macrophage in the reticulo-endothelial system, may be responsible for the abnormal morphology of red cells coated with IgG. The simultaneous deformation of a great number of red cells by a single mononuclear cell, rather

than the occasional phagocytosis of a few cells, may be a more efficient method of destruction. Deformed red cells, whether in the form of spherocytes or fragments, are susceptible to premature destruction in the unfavorable metabolic environment of the reticulo-endothelial system.

The mode of destruction of effete red cells in vivo is unknown. In this study, rosette formation did not occur with red cells aged in vitro. However, if aging in vivo results in the deposition of nonimmune IgG onto red cells, then monocytes or the macrophages could function to remove such red cells from the circulation. This method of destruction has in fact been suggested in experiments on mixed-cell systems (8), but there is no evidence yet that a similar phenomenon occurs in man.

#### SUMMARY

Red cells coated with IgG globulin were bound firmly to human mononuclear cells and formed rosettes. Rosette formation occurred when red cells were coated with IgG attached either immunologically (anti-D, anti-penicillin, or Donath-Landsteiner antibodies) or nonimmunologically with chromic chloride; no attachment was observed with cells coated with albumin. Rosette formation was blocked by pretreatment of white cells with sulfhydryl-binding reagents. Metabolic inhibitors did not prevent red cell adherence. White cells of other primates demonstrated a high degree of species specificity. Ultrastructural studies showed that the predominant leukocytes involved in rosette formation were monocytes, but some cells with characteristics of lymphocytes also formed rosettes. Considerable interdigitation of cell surfaces occurred at attachment sites and bound red cells appeared deformed. Thus, these studies confirm the presence of specific surface receptors for IgG on human monocytes and suggest that such receptors may provide a mechanism by which large numbers of red cells are eventually destroyed.

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